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Catalase Immobilized on Poly(Acrylic Acid-co-Vinyl Alcohol)

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Abstract

The paper studies the reaction of catalase's immobilization on a synthetic polymer (copolymer of the acrylic acid with vinyl alcohol), through amidation of enzyme's terminal amine groups with the lateral carboxylic group of the macromolecular support, as activated by dicycloxexyl carbodiimide. The support, possessing the properties of a hydrogel, has been synthesized through copolymerization of the acrylic acid with vinyl acetate, followed by hydrolysis of the acetate groups to the hydroxylic groups.

The influence of some reaction parameters (such as the activator/support and enzyme/support ratios, duration) on the efficiency of the enzyme's coupling was studied. The coupling reactions were realized conformely to a centered, rotator, composed, second order experimental program, which permitted the establishment of the conditions necessary for obtaining a coupling product containing the highest amount of immobilized enzyme.

Kinetic study of the reaction catalyzed by the coupling products has indirectly evidenced enzyme's immobilization to the macromolecular support, and also, a sufficiently high catalytic activity of the enzymatic preparation obtained.

Introduction

The advantages of employing enzymes immobilized on various supports – especially macromolecular ones – are well known, and they have been discussed in several studies [1-3]. When the support utilized is a polymeric one, biocatalyst's bonding may be achieved by several methods, which may be classified into 3 main groups, as follows [4]:

- crosslinking
- encapsulation
- chemical (ionic, covalent) bonding.

Support's selection – generally based on several criteria, such as the reactivity of its functional groups in moderate reaction conditions – and the immobilization method preferred – are especially important. Both natural polymers and their derivatives (mainly from the polysaccharide class) [5,6], and synthetic (hydrogels, especially) polymers may be employed as supports [7-11].

The present paper studies catalase's immobilization – through covalent bonding – on a copolymer of the acrylic acid with vinyl alcohol, in the presence of an activator, as well as the influence of some physical and chemical factors on the activity of the enzyme immobilized.

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Experimental

Materials

Catalase – extracted from bovine liver, molecular weight of 500,000

Macromolecular support – copolymer of the acrylic acid with vinyl alcohol (PAcA-VA) with a carboxylic group content of 3.5 Eq_{COOH}/g.

Activator – dicyclohexyl carbodiimide (DCCI).

Method

Synthesis of the support copolymer

Is performed in two stages, as follows: obtaining of a copolymer of acrylic acid with vinyl acetate, followed by the hydrolysis of the acetate groups to the hydroxylic ones.

35.5 mL vinyl acetate and 4.42 mL acrylic acid are dissolved in 160 mL benzene, together with 0.2514 g azo-izo-butyronitrile (AIBN, as initiator). The polymerization reaction is conducted into a balloon (vessel) equipped with a refrigerator, under reflux, for 3.5 h, at a temperature of 80°C. Benzene and the unreacted vinyl acetate are removed by distillation. The copolymer thus obtained is washed with warm water (5 portions of 200 mL each), for the removal of the unreacted acrylic acid and also of the poly(acrylic acid) formed, up to the pH of the distilled water, then dried in vacuum (40°C). There follows its dissolution in tetrahydrofuran (to obtain a solution of 3% concentration) for the removal of the unreacted vinyl acetate, precipitation in chloroform and, finally, drying in vacuum (40°C).

Transformation of the poly(vinyl acetate)-type structural units in vinyl alcohol is subsequently realized through hydrolysis of a copolymer solution in methanol (3%) with an alcoholic solution of NaOH. Precipitation of the reaction product occurs instantaneously, and separation of the new copolymer involves filtering, following by washing with methanol for the removal of NaOH (at a neutral pH). After drying, the copolymer is once again dissolved in warm water and then precipitated with concentrated HCl; finally, it is washed with water at pH = 7 and dried in vacuum.

For dosing of the carboxylic groups, a certain amount of copolymer is treated with 20 mL NaOH 0.1 N, for 5 h, by stirring at room temperature, followed by titration of the excess NaOH with a 0.1 N H_2SO_4 solution. This operation is performed for 5 samples, the results being exposed as average values.

Calculation of the number of gram equivalents of carboxylic groups per gram of copolymers applies the relation:

$$E_{COOH} = \frac{(V_0 \times f_{NaOH} - V_{acid} \times f_{acid}) \times 0.1}{10^3 \times m}$$

where:

- V_0 – volume of NaOH 0.1 N solution (20 mL)
- f_{NaOH} – factor of the NaOH solution
- V_{acid} – volume of the 0.1 N H_2SO_4 which titrates the sodium hydroxide remained unconsumed in the reaction with the polymer
- f_{acid} – factor of the H_2SO_4 solution
- m – copolymer weight taken into analysis.

The average content of carboxylic groups equivalents in the copolymer is: $E_{COOH} = 3.54 \times 10^{-3}$ Eq/g, which corresponds to a content of 25.5% (weight) and, respectively, to a molar content of 17.3% acrylic acid.

Catalase's coupling on the copolymer

0.2 g (a constant amount in all syntheses) copolymer is introduced in 15 mL disodic phosphate buffer 0.05 M (pH = 6.5), under stirring for 1.5 h, to assure the support's uniform dispersion. Then, still under continuous stirring, the volume of enzymatic liquid containing the amount of catalase established according to the experimental program is added. The activator (DCCI) dissolved in 2 mL THF – according to the experimental program – is added after 1.5 h – which represents the "zero" moment of the reaction. 20 mL phosphate buffer (0.05 M) are added, the final volume being constant for all syntheses performed. The reaction mixture is stirred as vigorously as possible at a temperature of 5°C, over durations established in the experimental program. When coupling reaction is over, the mixture is centrifuged for 15 mon (4,000 rpm) and, after supernatant's separation, 5 mL 0.2 M buffer phosphate (pH = 7) are added, then stirred and centrifuged. Washing is repeated 4-5 times, until the two phases are clear and the washing liquid is limpid.

The insoluble product separated is quantitatively passed into a 25 mL vial, 0.05 M buffer phosphate (pH = 7) being added up to a volume of 20 mL; the product is kept at a temperature of 4°C prior to the analysis.

Determination of the enzymatic activity

This involves iodometric titration, according to the followed method In 2 conic flasks of 100 ml each, 6 ml of sodium phosphate buffer 0.01 M at pH = 7 are measuring. In one of them is added 0.1 ml of enzymatic solution, and the other flask is used as blind sample. In both of flasks are added 0.2 ml of H_2O_2 3% concentration, stirring and then is maintained for exactly 5 min. at room temperature.

The action of enzyme on H_2O_2 is stopped by adding of 5 mL of H_2SO_4 solution (c = 10%) in each of them. After that 5 mL of KI solution (c = 10%) and one drop of ammonium molybdate (1% concentration) after added in each of flasks. The content of flasks is stirred carefully.

The released iodine from the reaction between KI and enzymatic undecomposed H_2O_2 is titrated with 0.02 N sodium thiosulphate until the solution will have

bright yellow colour. At this moment, 2-3 drops of starch solution (1% concentration) are added and the titration is continued until the colour is completely disappeared.

The difference between the two titrations (the sample and the blind sample) represents the amount of H_2O_2 decomposed by catalase's action.

As a unity of catalase's activity is considered the amount of enzyme which decomposes 1 μmol H_2O_2 (0.034 mg) in 1 minute. Because for 1 mL $Na_2S_2O_3$, 0.02 N solution corresponds 0.34 mg H_2O_2, it results that for the volume of $Na_2S_2O_3$, 0.02 N solution (representing the difference between the volume necessary for the titration of blind sample, *V*, and for the sample, *v*) corresponds:

$$x = (V - v)\cdot f\cdot 0.34 \text{ (mg } H_2O_2)$$

where:
- V – volume of $Na_2S_2O_3$, 0.02 N solution, consumed with reference sample's titration
- v – volume of $Na_2S_2O_3$, 0.02 N solution, consumed with sample's titration
- f – factor of the $Na_2S_2O_3$, 0.02 N solution (determined with $K_2Cr_2O_7$)

The amount of H_2O_2 decomposed in 1 min by the enzyme from 0.1 mL solution becomes:

$$x_1 = x/5\cdot 0.1 = 2\cdot x$$

Because 1 unity of catalase decomposes 0.034 mg H_2O_2/min, so *A* units of catalase will decompose x_1 (mg H_2O_2)/min. It results:

$$A = x_1/0.034$$

Finally, the enzymatic activity will be calculated with the relation:

$$A\ (\mu M\cdot min^{-1}\cdot mg^{-1}) = (V - v)\cdot f\cdot 20$$

Experimental program

Preliminary investigations have shown that the activity of the enzymatic preparation (which reflects, indirectly, the amount of immobilized enzyme) depends of series parameters, among which 3 are especially important: the reaction's duration, the enzyme/support and, respectively, activator/support ratio.

Correlation of the coupling products' activity with the above mentioned parameters might be attained with a function of the following type:

$$Y = a_0 + \Sigma a_i x_i + \Sigma a_{ij} x_i x_j$$

where:
- a_0 – free term
- a_i, a_{ij} – regression coefficients
- x_i, x_j – variables expressing the process parameters.

By means of a centered, rotator, composed, second order experimental design [12], the values of the regression coefficients were calculated.

The experiments were carried out according to the schedule given in Table 2. For the sake of simplicity in processing of results, the independent variables have been coded (Table 1).

Table 1
Variable's codification and their variation domain.

Real variable	Coded variable				
	-1.682	-1	0	1	1.682
Enzyme/support ratio (g/g) – x_1	8	21.7	36	50.3	64
DCCI/support (mol/mol_{COOH}) – x_2	1.1	1.262	1.5	1.738	1.9
Duration (h) – x_3	10	15 h 16'	23	30 h 44'	36

Table 2
Experimental program and obtained results.

No.	Coded variable			Activity of bonded catalase ($\mu M\cdot min^{-1}\cdot g^{-1}$)	
	x_1	x_2	x_3	real	calculated
1	-1	-1	-1	570	485.75
2	1	-1	-1	1365	1322.95
3	-1	1	-1	881	926.45
4	1	1	-1	1785	1824.26
5	-1	-1	1	813.75	791.85
6	1	-1	1	1785	1798.25
7	-1	1	1	1463	1401.55
8	1	1	1	2412	2469.15
9	-1.682	0	0	729	742.47
10	1.682	0	0	2520	2344.41
11	0	-1.682	0	723.15	770.19
12	0	1.682	0	1763.7	1705.04
13	0	0	-1.682	1130	1024.12
14	0	0	1.682	1795	1823.41
15	0	0	0	1824	1799.2
16	0	0	0	1803	1799.2
17	0	0	0	1768	1799.2
18	0	0	0	1795	1799.2
19	0	0	0	1817	1799.2
20	0	0	0	1788	1799.2

Starting from the experimental results obtained, the regression equation of the form:

$A = 1799.2 + 476.2x_1 + 277.9x_2 + 237.6x_3 + 15.3x_1x_2 + 42.3x_1x_3 + 42.25x_2x_3 - 90.4x_1^2 - 198.5x_2^3 - 132.7x_3^2$

could be established.

The high values of the coefficient of multiple correlation ($r = 0.966$) and of the F factor ($F = 40.57$) lead to the conclusion that the mathematical model proposed is suitable (as supported by the calculated values of the enzymatic activity – quite close to the experimentally determined ones).

Results and discussion

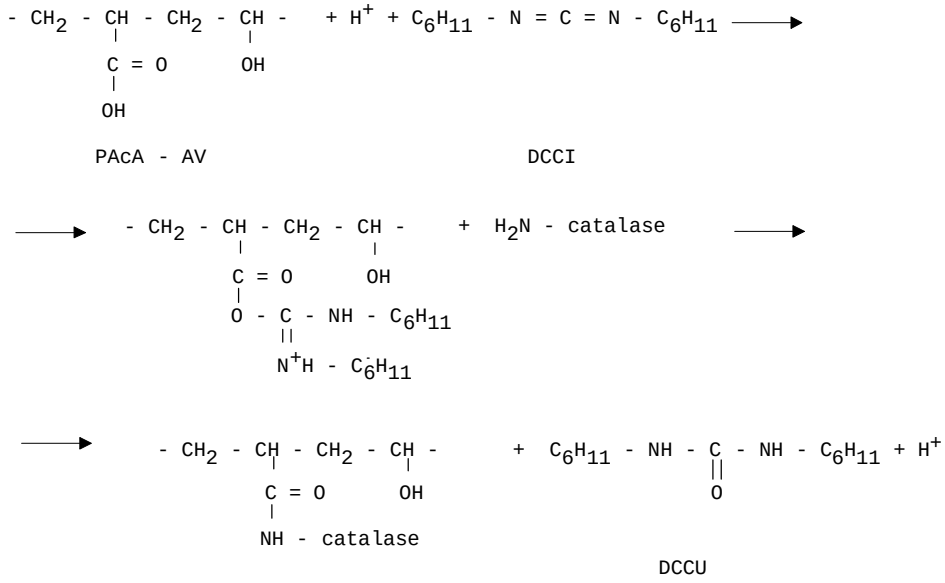
Synthesis of the macromolecular support followed the obtaining of a copolymer with high affinity towards the aqueous phase – in which enzyme's coupling occurs and within which it usually works.

IR spectral analysis has evidenced the absorption bands characteristic to the functional groups present on the support, at 1.710, 1.420, 1.320 cm^{-1} (-COOH group) and, respectively, 1.100-1.120 cm^{-1}.

Differential thermal analysis has evidenced, too, a curve of the weight losses with a unique decomposition stage, over the 120-420°C.

Over the 0-600°C interval, the maximum weight loss recorded is of 21.5%.

Enzyme's coupling to the macromolecular support, in the presence of DCCI as activator, is based on the following reaction:



With a view to establish the manner in which the parameters considered influence the reaction's efficiency (expressed indirectly by the activity of the preparations obtained), two variables (usually to the center of the experimental domain) have been particularized, which permitted the obtainment of some simplified functions which correlate the property had in view with the third variable.

Influence of the enzyme/support ratio

PAcA-VA is an insoluble polymer, which nevertheless swells in water, so that the coupling reaction is developed in a heterogeneous medium. The diffusion process is quite important in the reaction's development, so that enzyme's concentration in the substrate influences the coupling yield.

Analysis of the results presented in Fig. 1 evidences, indeed, an increased enzymatic activity of the coupling products with the increase of the enzyme/support ratio.

An increase of this parameter, on maintaining constant the amount of copolymer support, indicates an increase of biocatalyst's concentration in the reaction substrate, *i.e.* of the concentration gradient responsible for the intensification of diffusion inside the support's particles.

Influence of the activator/support ratio.

The diffusion process equally explains the increase of the coupling reaction's efficiency over a certain

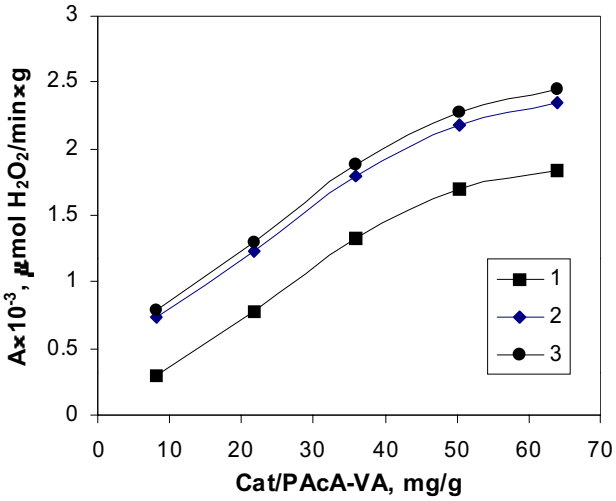


Fig. 1. Variation of the enzymatic activity of the coupling products with the enzyme/support ratio (mg/g). 1 – DCCI/PAcA-VA = 1.262 mol/mol$_{COOH}$; 2 – 1.5; 3 – 1.738 (t = 23 h)

variation interval of the DCCI/PAcA-VA ratio (Fig. 2).

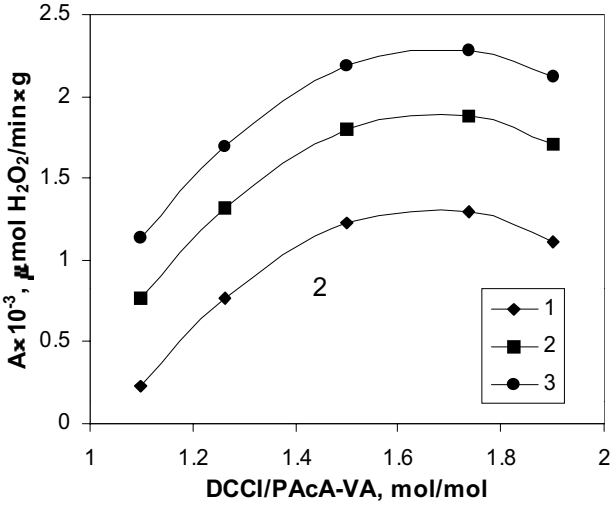


Fig. 2. Variation of the enzymatic activity of the coupling products with the DCCI/support ratio (mol/mol$_{COOH}$). 1 - Catalase/PAcA-VA = 21.7 mg/g; 2 – 36 mg/g; 3 – 50.3 mg/g (t = 23 h).

Increase of the reaction's efficiency is recorded up to a ratio of about 1.5 moles DCCI/mole –COOH, when diffusion's intensification with the increase of the concentration gradient facilitates the access of higher and higher amounts of activator to the support's carboxylic groups. For higher values of this ratio, the efficiency of coupling decreases, as a consequence of the fact that the excess of DCCI in the reaction medium favourizes reactions of intramolecular condensation or even crosslinking of the enzyme – which consumes the biocatalysts and takes it away from the reaction of coupling on the support. At the same time, the already immobilized enzyme may suffer crosslinking reactions that reduce its activity.

Duration

The activity of enzymatic preparations increases up to a duration of 20 h, which is the result of an increasing higher number of support's carboxylic groups capable of coupling the enzyme (Fig. 3).

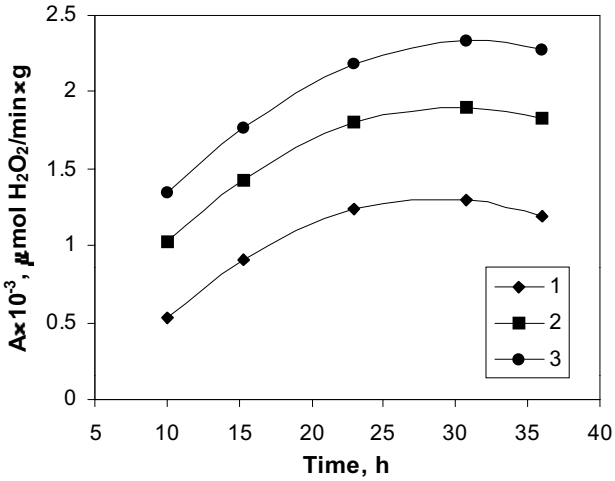


Fig. 3. Influence of the coupling reaction's duration on the activity of the immobilized enzyme. 1 – Cat/suport = 21.7 mg/g; 2 – 36 mg/g; 3 – 50.3 mg/g (DCCI/PAcA-VA = 1.5 mol/mol$_{COOH}$).

Decrease of the enzymatic activity at higher durations is most probably caused by some secondary reactions of enzyme's denaturation and, respectively, of its inter- and intramolecular condensation or crosslinking.

The correlated influence of two parameters of the immobilization process on the eficacity of coupling reaction is presented in Figs. 4 and 5. Once again, the conclusions presented above are proved.

In the absence of any absolute optimum as to the activity of the enzymatic preparations over the experimental domain taken into study, one may nevertheless appreciate that best results may be obtained if employing an activator/support ratio of 1.738, an enzyme/support ratio of 64 mg/g and, respectively, a duration of 30.44 h.

A product obtained under such conditions has been subsequently analyzed from the kinetic viewpoint of the reaction it catalyzes, as well as from the viewpoint of the properties characteristic to enzymes.

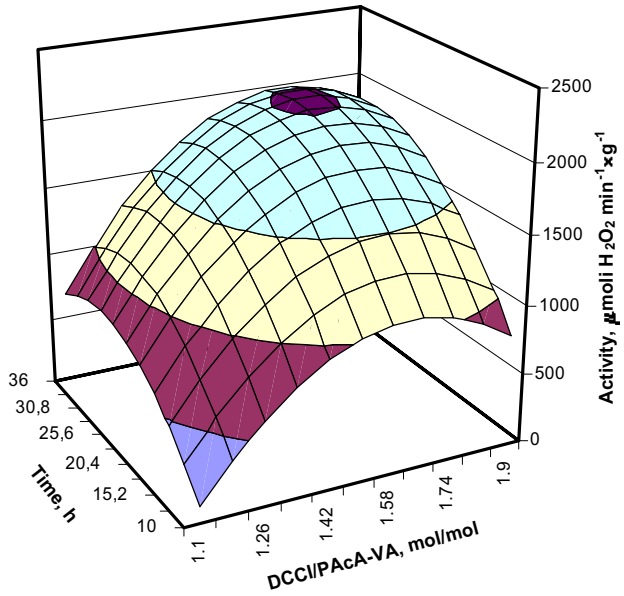


Fig. 4. The correlated influence of the coupling reaction duration and, respectively, the DCCI/support ratio (mol/mol_{COOH}) on the enzymatic activity of the immobilized enzyme (Catalase/PacA-VA = 36 mg/g).

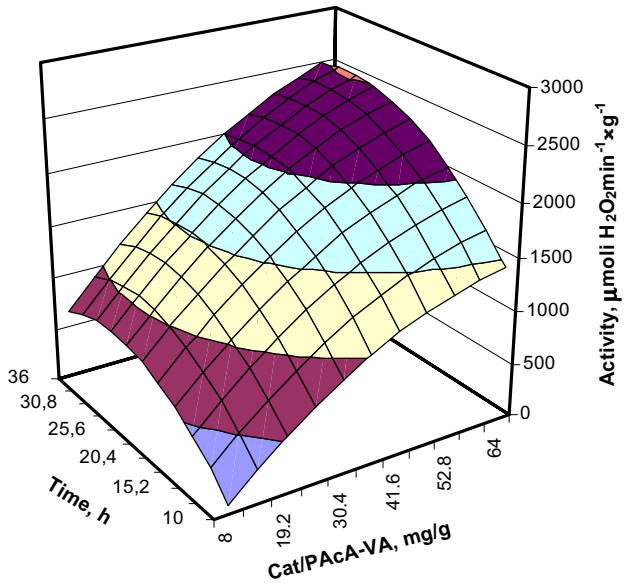


Fig. 5. The correlated influence of the coupling reaction duration and, respectively, the Catalase/PacA-VA ratio on the enzymatic activity of the immobilized enzyme (DCCI/PacA-VA = 1.5 mol/mol_{COOH}).

Influence of the enzymatic product's concentration

Generally, the rate of the enzymatically-catalyzed reactions is proportional to enzyme's concentration. On following this correlation for both the free and immobilized catalase, the results presented in Fig. 6 have been obtained.

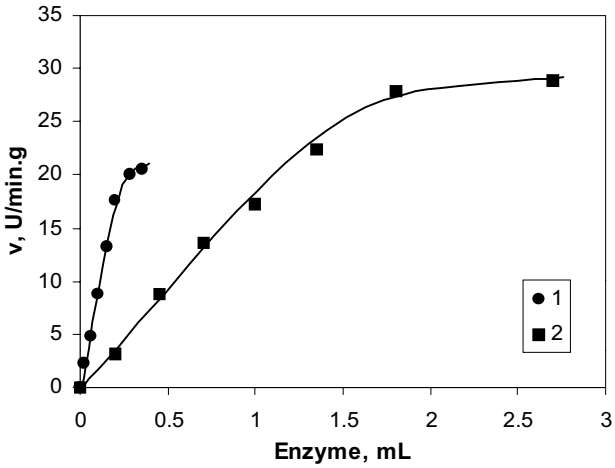


Fig. 6. Influence of the enzymatic product concentration on H_2O_2 decomposition rate. 1 - free enzyme 2 - immobilized enzyme. (Cata-lase/PacA-AV = 64 mg/g; DCCI/PAcA/VA = 1.738 mol/mol_{COOH}, t = 30.75 h).

It may be observed that the linear dependence between the reaction rate and the concentration of the enzymatic product is obeyed only for low and average values of the latter parameter. At higher concentrations, the decomposition rate of H_2O_2 decreases, which may be associated with the occurrence of some inter- and intramolecular interactions in the enzyme. The fact that the immobilized enzyme follows its linearity over a larger concentration domain may be explained by the fact that the molecules of the coupled enzyme have lower degrees of freedom as compared with those of the free enzyme and, consequently, lower possibilities of interaction.

Influence of the substrate's concentration

Enzymatic catalysis starts from the idea that, between enzyme *E* and substrate, *S*, an intermediary complex, *ES*, is forming, which may be either dissociated in its initial substances or split into enzyme and product, *P*, according to the general reaction:

$$E + S \leftrightarrow ES \rightarrow E + P$$

Mathematical modeling of such reactions leads to the Michaelis-Menten equation:

$$V = V_{max} \times [S]/([S] + K_M)$$

where:
- V – reaction rate
- V_{max} – maximum reaction rate
- $[S]$ – substrate's molar concentration
- K_M – Michaelis Menten constant.

The graphical representation of this dependence,

in the case of enzymatic preparations based on the catalase immobilized on PAcA-VA is presented in Fig. 7.

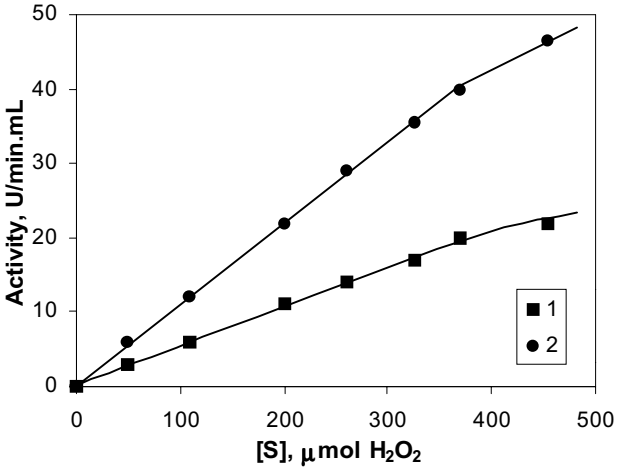


Fig. 7. Variation of the activity of the enzymatic preparations with the substrate's concentration. 1 – immobilized enzyme. (Catalase/PAcA-VA = 64 mg/g; DCCI/PAcA-AV = 1.738 mol/mol_{COOH}, t = 30.75 h); 2 – free enzyme.

The observation to be made is that, at low H_2O_2 concentrations, catalase's activity increases proportionally with the number of moles from the reaction medium. At high substrate concentrations (curve 1), enzyme's activity attains a maximum value, followed by a slight decrease, which might be explained (as stated in the literature of the field, as well) [13], by the inhibitory effect exercised by high H_2O_2 concentrations, on catalase's activity.

For the characterization of an enzyme, especially important is to know the value of Michaelis-Manten constant (K_M), which reflects its affinity toward a specific support. Its determination is made by means of the Lineweaver-Burk representation (Fig. 8).

It is observed that, through its immobilization to the polymeric support, the value of K_M increases about 2 times, comparatively with the free enzyme.

Such an increase and, respectively, a decrease of the immobilized catalase's affinity towards the substrate, may be explained by the hindering of the substrate's diffusion, as a result of immobilization, as induced by steric hindrances and also by the electrical barrier [14].

Elimination of such negative effects and also obtaining of a K_M value of the immobilized biocatalyst closer to that of the free one involves stirring or the reaction mixture or increase of the enzymatic preparation's concentration.

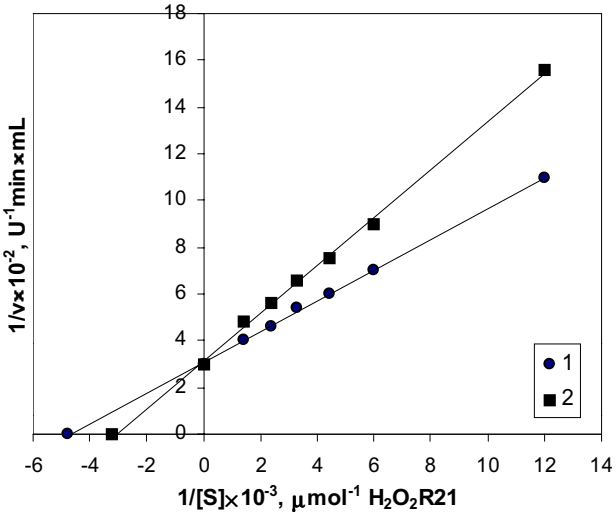


Fig. 8. Determination of the Michaelis-Menten constant for both the free (1) and the immobilized (2) enzyme. (Catalase/PacA-AV = 64 mg/g; DCCI/PAcA-VA = 1.738 mol/mol_{COOH}; t = 30.75 h).

Conclusions

Catalase may be immobilized through chemical bonding to poly(acrylic acid-co-vinyl acetate), in the presence of dicyclohexyl carbodiimide, which leads to an insoluble, catalytically active preparation.

The efficiency of the coupling reaction depends on the reaction's duration, enzyme/support and, respectively, activator/support ratio.

Catalase's immobilization on the copolymer is followed by an increase of Michaelis-Menten constant's value, versus the free enzyme.

The influence of certain external factors (such as, concentration of the enzymatic preparation, substrate's concentration, *etc.*) is similar on both the free and immobilized enzyme.

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